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Tissue tension and not interphase cell shape determines cell division orientation in the *Drosophila* follicular epithelium

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1st Editorial Decision 26th Jul 2018

Thank you for submitting your manuscript entitled 'Tissue tension and not interphase cell shape determines cell division orientation in an epithelium' to The EMBO Journal. We have now received three referee reports, which are included below. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version

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REFEREE REPORTS:

Referee #1:

The manuscript by Finegan et al. addresses the mechanism by which cell division is oriented in the Drosophila follicle epithelium covering the egg chamber. It also addresses the question whether cell division is required for the elongation of the egg chamber. The authors show that blocking cell division does not affect egg chamber elongation. Furthermore, the authors use laser ablation experiments to probe tension in the follicle epithelium and claim that tension is anisotropic with higher tension along the long axis of the egg chamber. As shown previously, the long axis of cells is preferentially oriented perpendicular to the long axis of the egg chamber. Furthermore, the authors provide evidence that cell division orientation is biased in a way that cells divide along the long axis of the egg chamber. Orientation bias depends on canoe, but not on Mud (a protein previously shown

to help determine division orientation). The authors conclude that not cell shape (or distribution of TCJs), but tissue-scale tension orients cell division in the follicle epithelium.

Understanding how cell division is oriented relative to a tissue axis is important for understanding tissue formation and function. The major claim of this study that tissue tension, and not interface cell shape, determines cell division orientation is not well supported. The authors fail to convincingly show that there is a tissue-level anisotropic stress in the follicle epithelium. The bias in cell division orientation, moreover, seems to be rather weak (if there is any; a recent paper by Alegot et al, 2018 failed to see it). The dependence of this weak bias on Canoe is of some interest. However, Canoe protein is uniformly localized to the cell cortex, and thus it is unclear how Canoe could have an instructive (rather permissive) function in cell division orientation. Finally, direct evidence that anisotropic tissue tension is required for oriented cell division is not provided.

The manuscript is not particularly well written. It lacks focus reporting too much data that is not directly relevant to the main question of the manuscript (e.g. Figs. 1-3). Moreover, conclusions are sometimes overstated and statistical analysis is missing in numerous cases.

Finally, a role of tissue tension in directing cell division orientation has been recently reported by Wang et al. 2017 for mesectoderm cells in the Drosophila embryo (Finegan et al. cite this paper).

In summary, the study addresses an important question, but fails to provide conclusive data to support its major claims.

Major concerns

-Fig. 3C: The authors claim that Sqh localization changes from a cell-level organization to a higher order level organization between stages 3 to 6. To the eyes of this reviewer, there is no qualitative difference in Sqh localization between these stages. It is also difficult to tell whether Sqh is cortical or not in the absence of a cortical marker.

-Fig. 4C. Is there a statistical difference between control and string knockdown? N for the knockdown seems to be quite low as compared to the control.

-Fig. 4F,G. It is unclear to me what mechanical tension the authors aim to measure. Laser ablation experiments, like the one described here, are sometimes used to measure mechanical tension along individual cell junctions. For this, a single junction is cut by focused laser light. The recoil/displacement of the two vertices of the cut junction is then used as a proxy for relative tension on cell junctions. In this figure, however, I do not see that the authors cut a single cell junction, nor do I see the vertices of junctions. Instead, the authors seem to have measured the displacement of the cut ends of the cortex. The displacement of the cut ends of the cortex may give some insight on the properties of the cortex, but I do not see that it is a proxy for tension on cell junctions.

-Fig. 4H,I. How did the authors define the 'edge' of the ablated region? This is crucial to get precise measurements of ratios. Is the deviation of the recoil ratio AP/UD significantly different from 1?

-Fig. 5A. It is not obvious to this reviewer that cell division orientation is biased in relation to the long axis of the egg chamber. To assess significance, the experimentally found distributions should be statistically compared to a random distribution of angles.

-Fig. S5E. Since the cell division orientation bias is not obvious in the control situation, it is unclear whether such a bias is 'lost' in the fat2 mutant, as claimed by the authors. Statistical analysis as suggested above is required. In addition, the authors claim that cell division orientation is biased by tissue-level tension, based in part on the claim that tissue-level tension is reduced in rounder egg chamber (like fat2 mutants). What is the evidence that tissue-level tension is reduced in rounder egg chambers?

-Fig. 5D. This is one of the most important experiments, but as it stands is unconvincing. The

authors need to increase the number of analyzed cell divisions (now it is nine) and should plot the distribution of angles (e.g. in a rose diagram). Hertwig rule does not mean that all cells divide exactly perpendicular to their interphase long axis, but that there is a bias towards this division orientation. The question is therefore how strong (or weak) this bias is in the follicle epithelium as compared to other epithelia that have been previously analyzed (e.g. Bosveld et al, 2016).

-Fig. S5F It is unclear how the conclusion that the distribution of cell vertices does not correspond to division orientation is reached.

-Fig. 6A,B. The authors claim that Mud (the authors in fact look at Mud-GFP) localize around the entire mitotic cell cortex in the follicle epithelium, in contrast to the pupal notum where Mud is enriched at tricellular junctions (yet detectable around the entire cell cortex (Bosveld, 2016)). The pixel intensity appears saturated in Fig. 6A,B and I am wondering whether the authors are therefore missing an enrichment at tricellular junctions. Better images are required.

-Fig. 6D. I suspect that the authors have plotted the angle of spindle rotation. The authors state that they have sometimes observed oscillatory behavior. How is the angle of spindle orientation defined under such behavior? Moreover, I cannot follow the authors' argument that differences in the magnitude of spindle rotation suggests differences in the way the orientation of division is determined.

-Fig. 7B. The authors need to test whether canoe shRNA specifically targets canoe, e.g. by using at least two additional independent shRNA lines. Or even better, but perhaps more difficult, to analyze planar division orientation in cno-R2 mutant clones that the authors have generated. Moreover, is the angle distribution between wt and random number generator significantly different?

-The authors would need to show some more direct evidence that tissue tension directs cell division orientation. Wang et al 2017, for example, used laser ablation to mechanically isolate cells in the Drosophila embryo. These cells normally divide in an oriented fashion, but when mechanically isolated divide with random orientation. Similar experiments would need to be done in the follicle epithelium.

Minor concerns

- Fig. 1C: The authors claim that cells are consistently smaller by stages 6-7. However, smaller appears to relate to apical/basal area, not volume (cells could change their height). The conclusion that proliferation outpaces elongation (of the egg chamber?) seems therefore not adequate.

-Page 6 top: What does 'loosely organized state" mean? The authors need to be more precise.

- Page 6 top: "Together, these results show that the FE is proliferative and dynamic.." These conclusions are quite general and not novel. I do not see evidence that "the material properties of the tissue change..." The authors should formulate the conclusion more precisely. Moreover, two paragraphs later, the authors write "The relatively static arrangement of cells..." Wording needs to be improved.

Fig. 2A,B. The authors claim that knockdown of string does not affect egg chamber elongation. The authors should measure the aspect ratio for string knockdown and control egg chambers for different stages.

-Fig. 2C: Does the cell shape regularity differ between controls and string knockdown? Statistical test is required.

-Fig. S2C: I do not see a gap where the arrow is pointing to.

-Fig. 3C: The distribution of Sqh at the basal site of cells, as shown in the lower panels, is not mentioned in the results section.

-Page 10. The authors claim that the rate of proliferation is constant over the tissue, but do not show data. Either the authors show the data or do not make this claim.

-Fig. 5B. The authors claim that dividing cells detach and are excluded from the basal surface. I do not see this. In fact, in the basal view there is a tiny cell in the center of the image. Is this the basal foot of the dividing cell? The authors need to label single dividing cells (e.g. by expressing GFP in single-celled clones) to clarify this issue.

-Fig. 5C. The authors claim that the cell shape bias is the same at apical and basal cell surfaces. This panel shows a segmentation, but I do not see that apical and basal surfaces of a cell are compared.

Suggestions

-Abstract: The abstract should include a few sentences of background information.

- Title: The authors investigated cell division in the Drosophila follicular epithelium. This should be indicated in the title.

- Possible typos:
- page 8 bottom: "This raises suggests.."

Referee #2:

In this manuscript, Finegan and colleagues investigate the role of cell divisions in the follicular epithelium (FE) in the morphogenesis of the egg chamber in Drosophila. The authors use take advantage of the genetic tractability of Drosophila, and use both fixed and live tissue imaging, quantitative image analysis and computer modelling, in a technical tour de force that reveals that oriented cell divisions contribute to optimal cell packing. The authors found that, as the egg chamber expands, follicle cells divide and get smaller, while also getting more regular (hexagonal) in shape. Inhibiting cell division in the FE did not affect egg chamber elongation, but eventually led to gaps in the FE. In addition, when division was inhibited, cells did not become more regular in shape over time. The authors did not find significant changes in junctional (beta-catenin) or cytoskeletal (myosin II) protein levels during the course of egg chamber elongation. However, they found that the distribution of the myosin light chain changed apically, transitioning from a cortical localization into a meshwork of nodes and fibers. Inhibiting cell division resulted in FE cells with greater apical area, stretched along the axis of tissue elongation, and thinned down along the equator, suggesting that the FE is stretched to cover the elongating egg chamber. Using laser ablation, the authors suggest that tension was greatest in the equator than at the poles, and that there was preferential tension along the axis of tissue elongation. As the chamber expanded, cell division orientation became better aligned with the long axis of the chamber. But during interphase, FE cells were oriented perpendicular to the long axis of the chamber. Interestingly, this contradicts Hertwig's Rule, which postulates that cell division orientation correlates with interphase cell shape orientation. Using fat-shRNA, which results in somewhat rounder chambers, the authors found that the cell division orientation was lost, attributing this effect to changes in tissue tension. Vertex distribution during interphase, which was recently shown to predict division orientation in wing discs, was not predictive of division orientation in follicle cells (consistent with the localization of Mud, which orients the spindle with Dynein, throughout the cortex and not in vertices). The spindle rotated both in FE cells and in the embryonic ectoderm (both "immature" epithelia in which Mud is not at tricellular vertices). Furthermore, Mud loss of function did not affect planar cell division orientation. In contrast, loss of the adaptor protein Canoe resulted in myosin detachment from the cortex, as previously reported in embryos, and randomized division orientations (but did not affect tissue elongation). However, inhibition of the myosin activator Rok did not affect cell division orientation. A mathematical model predicted that biasing the orientation of cell division along the direction of tissue stretch maximizes the number of hexagonal cells when compared to the cell division orientation predicted by interphase shape or randomized division orientations. Based on this, the authors propose that tension-driven division orientation contributes to regular cell packing.

The work presented here is interesting, as it introduces a model that disrupts Hertwig's rule, and provides some hints about other mechanisms that may orient cell division. The work is generally very well quantified, and the authors should be commended for that. However, the mechanistic aspects of the work are confusing: the evidence for the role of Canoe/myosin in orienting divisions is contradictory, and the importance of packing the cells into a hexagonal array is not clear. There are also some results in which the interpretation provided by the authors requires additional validation. Thus, I suggest that the authors address the following points before further considering the manuscript:

MAJOR
1. The authors show that inhibiting Rok with Y-27632 does not affect cell division orientation. They should try to further increase the concentration of Y-27632 until they observe myosin displacement from the cortex, and thus can confirm that a) myosin is not necessary for cytokinesis, and b) myosin does not affect division orientation. Otherwise, how do they reconcile the fact that cell division orientation is reduced in cno loss-of-function, but not in Y-27632? If not through myosin, how does then Canoe control cell division orientation? This is a major outstanding issue.

2. Figure S5D-E: I have several issues with these figures:
a. The rounding of Fat2 shRNA is significant, but the chamber is still significantly elongated. Are there other treatments that result in rounder chambers and could be used to confirm these results? Fat is a key component of the planar cell polarity (PCP) pathway, and I worry that the phenotype in cell division orientation could be due to a PCP defect.
b. What is the evidence that cell division orientation in Fat2 shRNA is independent of cell shape cues? In Fat2 shRNA chambers, the cell shapes also lose their preferential orientation perpendicular to the long axis of the chamber, right?
c. The authors claim that anisotropic tissue tension is lost in Fat2 shRNA, but they should use their laser ablation system to demonstrate this. Based on the significant elongation of Fat2 shRNA chambers (Fig S5D), it is likely that tension is still anisotropic.

3. The authors use laser ablation to probe the mechanical properties of the apical surface of FE cells. It was not clear to me, based on the description of the methods, whether they are using a two-photon system (it could be, based on the wavelength used for ablation). Regardless, because apical is "deep" in this tissue, and equator cells are flatter than pole cells, it is possible that the differences in recoil velocity could be explained by a difference in the ability of the laser to reach the apical surface of polar vs. equatorial cells. Given that the authors find similar cell shape changes apical and basal (Fig 1D), they should try to confirm their results conducting laser cuts on the basal surface, which should remove any concerns about laser penetration.

4. Figure 3A-B: using what seem like low magnification images of egg chambers, the authors argue that the levels of beta-catenin and non-muscle myosin do not change in the FE as the chamber elongates. However, there is no quantitative analysis to support that claim. In fact, based on Figure 3B, I would argue that I first see an increase and then a decrease in apical myosin fluorescence. The authors should provide quantitation of beta-catenin and myosin fluorescence to back up any statements about levels.

5. What is the importance of hexagonal packing? How beneficial (and why) is it for the embryo to obtain the relative increase in hexagons observed in Figure 8B (left) with respect to what you would get if divisions followed Hertwig's rule (Figure 8B, center), or were randomly oriented (Figure 8B, right)? In other words, what is the advantage of the final cell packing in wild-type chambers vs. cno shRNA? This should at least be discussed.

MINOR
1. Figure 3C: are these cells in the poles or the equator of the egg chamber? Are there any differences in the distribution of apical myosin between polar and equatorial cells? This is important for the interpretation of the laser ablation experiments.

2. Figure 4D, E: the authors report the ratio of polar/equatorial cell heights, but it would be useful to see the absolute values of these heights to really understand how much is the tissue flattening.

3. Figure 5A: the authors plot the orientation of cell division with respect to the long axis of the

embryo (i). But wouldn't it make more sense to plot the orientation of cell division with respect to the axis of tissue expansion (shown in ii). Wouldn't those correlations show the bias of cell division orientation in the direction of tissue expansion a lot better?

TYPOS
1. Page 4, paragraph 3: the reference to (Fig 4F, G) should be a reference to (Fig 4D, E).

2. Page 4, paragraph 3: "This raises suggests two possibilities" has too many verbs!

3. Page 14, last sentence: "Adherens junctions are not proximal to spindle poles in this tissue (Fig S4C)". Fig S4C shows a recoil speed after laser ablation. Is that the correct figure reference?

4. Page 15, paragraph 2, line 6: should the reference to Fig 5A be a reference to Fig 7B?

5. Page 22: please review this sentence "Cuts in the saggital view were performed on an Olympus FV1000MPE and an Olympus Fluoview FVMPE-RS Twin Lasers Gantry System using an Olympus 25x 1.05NA objective lens which using an Insight X3 laser."

Referee #3:

The proper regulation of organ shape is a key question in cell and developmental biology. Cell proliferation, migration, and shape change all have the potential to play important roles. The Drosophila ovary has emerged as a superb model, and analysis of its morphogenesis has already revealed a number of very striking features, including a fascinating collective migration event. Building on this foundation, the authors explore earlier events in ovary morphogenesis. They use a very impressive set of quantitative approaches to comprehensively assess the contributions of proliferation, migration and oriented cell division to the final ovariole shape. They find that cell proliferation and increased packing increases cell shape regulatory, and identify a surprising and exciting role for tissue wide biases in myosin-based tissue tension in directing oriented cell division in a way that defies Hertwig's rule, which governs cell division in most contexts. They couple direct observation with mathematical modeling to assess this hypothesis. The results are well documented, carefully quantified, and support their conclusions. I think this story, if suitably revised, will be of broad interest to cell and developmental biologists.

While most of the data was quite convincing, there were a couple of places where the data or its analysis could be solidified. More important, the authors present an exceptionally comprehensive analysis-a mountain of data. However, perhaps because of space limitations, this means that a number of the assays and results are described so succinctly as to make them difficult for the average reader (me, in other words), to follow. I note these below. All should be relatively straightforward to address, and if more space is required I'd encourage the editor to allow this.

Major issues

p. 5 and onward. Throughout the authors assess parameters of cell shape, but in the text do not tell us which molecule they were visualizing,

Fig. 2. The authors state that string knockdown does not change aspect ratio but do NOT actually show the data. A graph of aspect ratio should be added. They also state that "proliferation outpaces growth" and bolster this by assessing cell area-however, without an independent assessment of cell height (which I imagine they made), I am not sure how they can conclude this.

p. 7, last paragraph. The authors measurements of Arm and myosin protein levels do NOT, by themselves "refute the possibility" that there is an increase in junctional tension. This needs to be tempered.

The one place where the data did not match the conclusions was in their assessment of planar polarity of important proteins. They present images of Ecad, Cno and Jub and state there is "no

obvious planar polarity". These data have two issues. First, in the image they show Jub looks clearly planar polarized . Second, planar polarity is seldom "obvious". They should quantify levels of each on A/P vs. D/V borders. In the extending germband, for example, planar polarity of Ecad is less than 2 fold, and not immediately obvious by eye. I think these potential differences are very interesting and fit with their later analysis of rhe role of Cno.
Fig. 4D,E should also include a graph of actual cell height as well as the ratio shown.

Fig. 4F-I and p. 9 middle. I was confused by the explanation of the laser cutting experiments. In the text the authors suggest they could not measure "recoil speed" yet that is exactly what is reported in Fig. 4G. This needs to be better explained.

p. 12, top. The quantification of divisions live reported here was important but the authors need to describe what the data mean-what would be the predictions if there was no bias and how do these data refute that.?

p. 13 and Figure 6. I found the description of the vertex model confusing. I also though some of their assertions were not accurate In Fig 6A Pins appears to be enriched at vertices, while they suggest otherwise.

I also had trouble with their descriptions of the events around spindle rotation. Do their data rule out a model where a cortical cue captures spindle poles as the cell transitions from metaphase to anaphase? I think the conclusions in this section are too strong.

p. 15, top. The authors saw no effect for Ecad KD, presumably due to Ncad's presence. Did they try Arm KD?

p. 16, Fig 8. The use of mathematical modeling is a significant strength. However, the brevity with which this data is described makes it difficult to assess the match of model and data.

Text and data clarity issues

p. 4. The authors first introduce Hertwig's Rule and the Tricellular junction rule here, but do not define either for the reader who is not in the field.

p. 5, top. In the 1st sentence of the results the authors should be clearer, stating that they examined geometry "during the proliferative stages, as the egg chamber aspect ratio increases from ~1.0 to 1.6" and then annotate this on Fig. 1A.

Fig. 1C-E, p. 5 middle. Here is where the authors begin their quantitative analysis. They need to go much more slowly, explaining to the reader what they measured and what it means. These data underlie one of their important conclusions-that proliferation increases cell shape regularity.

Fig. 3C is very interesting and important yet complex. It needs more interpretation for the reader, annotating cell border and apical staining. I'd also like to see a still from stage 6 in which both myosin and a junctional marker were included to see how the supercellular structures align with cells. Finally, it would be better to conclude that the changes in Sqh localization "coincides with" rather than "corresponds to" the change in tissue regularity

Fig. 4A-C is described in a single sentence!

p. 9, top paragraph. I had a difficult time following the logic in which observations of uneven cell height led to the conclusions about mechanical deformation-explain better or remove.

p. 10, middle. Where is the data reporting PH3 immunoreactivity mentioned here?

p. 10, bottom and Fig. 5A. These data are key to one of the conclusions and once again need to be more thoroughly explained. Define what 0 degrees means-perhaps with a vector diagram of zero versus 90 degrees added to the top of this panel/. . Place a line at 45 degrees as that would be the predicted mean of random divisions.

P 12, middle. The section starts with a bold statement about the uncoupling of cell shape and tension that I was not able to simply connect to the preceding data.

p. 14, bottom. Canoe ≠ adherens junctions, and the sentence should read "Canoe is not enriched proximal to..." (and the ref. should be to Fig. S6C).

p. 15, bottom. Please do not describe the effect on myosin with the term "drops away". Earlier authors have described this as an effect of Cno loss on the linkage of actomyosin to cell junctions.

p. 16, top. The logic of this paragraph was totally lost on me.

Minor issues

p. 4, top. The FE rotates, while the underlying germline does not, so I would be careful to not simply say the "egg chamber rotates". Likewise, state the divisions are oriented "perpendicular to the apical-basal axis".

p. 5, bottom. Cells do not "get smaller"-apical area does.

Fig. 2A. Tell us what stage is displayed.

p. 8, bottom. In the last paragraph a Figure reference is incorrect-it should be "anterior and posterior poles (Fig. 4D,E)."

p. 13, top. Temper this conclusion. "This observation may be explained by..."

p. 15. Several Suppl. Fig refs. here are wrong.

1st Revision - authors' response 24th Sep 2018

We are grateful to the editor and referees for their careful consideration of our manuscript. We have addressed the specific reviewer concerns as follows:

Referee #1:

Major concerns

-Fig. 3C: The authors claim that Sqh localization changes from a cell-level organization to a higher order level organization between stages 3 to 6. To the eyes of this reviewer, there is no qualitative difference in Sqh localization between these stages. It is also difficult to tell whether Sqh is cortical or not in the absence of a cortical marker.

To address the reviewer's concerns, we have now included images of Sqh together with the membrane marker Shg (DE-Cadherin). To address the reviewer's request for evidence that Sqh is cortical we have added an image of an egg chamber imaged in sagittal view showing that Sqh localizes specifically to the apical cortex, colocalizing with Shg (Fig EV6E) (consistent with previous reports, including Wang and Reichmann (2007) and Alégot et al (2018)). We have clarified the localization of Sqh at the apical cortex relative to cell-cell junctions by the addition of a new figure of Sqh-mCherry and Shg-GFP at the apical cortex through developmental time (Fig 3C). To strengthen our claim that Myosin-II exhibits a change in organization at stage 6, we have added a higher-resolution confocal image of Zipper-YFP (Myosin heavy chain) at the apical cortex (Fig 6A) demonstrating that Zipper exhibits the same localization pattern through development as observed for Sqh-GFP.

-Fig. 4C. Is there a statistical difference between control and string knockdown? N for the knockdown seems to be quite low as compared to the control.

The String knockdown egg chambers have fewer cells per egg chamber than the control, and the total number of cells analyzed is therefore much lower. We regret that the statistical difference between the wt and experimental conditions were not made explicit in our original submission. That was an oversight on our part. Using a Mann-Whitney statistical test, the *p* values for the original data are 0.0067 at AR 1.2-1.4 and <0.0001 at AR 1.4-1.6. We have included them in the resubmission.

-Fig. 4F,G. It is unclear to me what mechanical tension the authors aim to measure. Laser ablation experiments, like the one described here, are sometimes used to measure mechanical tension along individual cell junctions. For this, a single junction is cut by focused laser light. The recoil/displacement of the two vertices of the cut junction is then used as a proxy for relative tension on cell junctions. In this figure, however, I do not see that the authors cut a single cell junction, nor do I see the vertices of junctions. Instead, the authors seem to have measured the displacement of the cut ends of the cortex. The displacement of the cut ends of the cortex may give some insight on the properties of the cortex, but I do not see that it is a proxy for tension on cell junctions.

As described in the manuscript, we measured cortical tension (rather than junctional tension) by making laser cuts in the apical cortex. This approach has substantial precedence in the literature. Published examples include: 1) Lye, C.M. et al., 2015. Mechanical Coupling between Endoderm Invagination and Axis Extension in Drosophila. *PLoS Biology*; 2) Collinet, C. et al., 2015. Local and tissue-scale forces drive oriented junction growth during tissue extension. *Nature Cell Biology*; 3) Chanet, S. et al., 2017. Actomyosin meshwork mechanosensing enables tissue shape to orient cell force. *Nature Communications*; 4) Hara, Y., Shagirov, M. & Toyama, Y., 2016. Cell Boundary Elongation by Non-autonomous Contractility in Cell Oscillation. *Current Biology*. These papers describe ablation of the cortex on a scale larger than a single junction, with recoil analysis performed via imaging of the surrounding cortex to read-out cortical tension. Therefore, we agree that our experiments provide insight into the tension of the cortex and not single cell junctions.

-Fig. 4H,I. How did the authors define the 'edge' of the ablated region? This is crucial to get precise measurements of ratios. Is the deviation of the recoil ratio AP/UD significantly different from 1?

We defined the edge as the boundary between pixel intensity and the lack thereof, which is typical for these experiments. We have added arrows to the images in Figs 3F and 3H to indicate the edge of the ablated regions. A Wilcoxon Signed Rank Test for significance of the AP/UD recoil ratio reveals that the median is significantly different from 1. We have added the *p* value (0.01577) to the Figure.

-Fig. 5A. It is not obvious to this reviewer that cell division orientation is biased in relation to the long axis of the egg chamber. To assess significance, the experimentally found distributions should be statistically compared to a random distribution of angles.

We described a randomly generated distribution of division angles in our original submission (Figure 7B, now in Figures 5 and 7), and have included it (unchanged) in the resubmission. As stated in the text of the original submission (page 14), the wild type "division angle data [is] significantly different from a hypothetical random data set." In our manuscript, we chose to emphasize these data, which were generated using an unbiased software approach, over the data in 5A (now 4B), which was generated manually by two experimenters. (We note that the two data sets are equivalent, indicating that both methods are sound).

The reviewer is correct that statistical significance was not described for these data. The *p* values for our manually-collected data at Stage 6 are 0.0031 (Wilcoxon Signed Rank Test, Theoretical Mean of 45) and 0.0218 (comparison to a randomly generated list of the same size). We have amended the figure legend accordingly.

The reviewer also noted in their summary that Alégot *et al* did not measure a division orientation bias in their work. "The bias in cell division orientation, moreover, seems to be rather weak (if there is any; a recent paper by Alegot et al, 2018 failed to see it)." We discussed this point in our original submission. The bias we measured is significant at Stage 6, during which period divisions are relatively infrequent, and corresponds to the weak directional expansion of the egg chamber that occurs at that stage. Developmental stage is not accounted for in the previous study by Alégot et al, and the bias in spindle orientation would therefore be disguised.

We are pleased to note that the bias we report has been independently corroborated by Dong-Yuan Chen *et al,* as described in a preprinted article made available on bioRxiv: (https://www.biorxiv.org/content/early/2018/08/06/384958) during our revision period. A citation to the preprint has been added to our manuscript following our discussion of the Alégot et al results.

-Fig. S5E. Since the cell division orientation bias is not obvious in the control situation, it is unclear whether such a bias is 'lost' in the fat2 mutant, as claimed by the authors. Statistical analysis as suggested above is required. In addition, the authors claim that cell division orientation is biased by tissue-level tension, based in part on the claim that tissue-level tension is reduced in rounder egg chamber (like fat2 mutants). What is the evidence that tissue-level tension is reduced in rounder egg chambers?

Division orientation is statistically different from random in wild type egg chambers at Stage 6. It is not different from random in Fat2-shRNA egg chambers at Stage 6, which are somewhat less elongated than wild type. We have added another condition, Pak-shRNA, in which the egg chambers are much rounder than wild type and division angles are significantly different from wild type.

We have no evidence, and do not claim, that the magnitude of tissue-level tension is reduced in rounder egg chambers. Rather we suggest that anisotropy in tension across the tissue is reduced. We have performed ablation experiments on younger egg chambers (stages 4 and 5) which are rounder, analyzing those with aspect ratios of less than 1.3. We found that unlike in stage 6 egg chambers, recoil in the elongating (AP) versus the rotation direction (UD) was equal on average, consistent with our model (Mean ratio of recoil AP vs. UD = 0.9871; Median = 0.9884; Wilcoxon Signed Rank Test compared to theoretical median of 1 $p = 0.8438$). These data, now presented in Fig EV3E, indicate that tension anisotropy is reduced in rounder egg chambers, consistent with our model and spindle orientation results.

We would ideally like to measure tissue tension in rounder egg Stage 6 chambers by performing laser ablation on chambers that have been genetically modified (for example *fat2* or *pak* mutant egg chambers). This experiment requires genetically complex fly lines containing 4 distinct elements: sqh^{AX3} allele to remove endogenous Sqh, transgenic sqh-GFP to label Sqh for imaging, Traffic Jam-Gal4 to provide a driver for shRNA expression in the follicular epithelium, and the UAS-shRNA driving transposable element. We attempted to generate these flies but were unable to recover ovaries. We attribute this problem to the complexity of the genetic background.

-Fig. 5D. This is one of the most important experiments, but as it stands is unconvincing. The authors need to increase the number of analyzed cell divisions (now it is nine) and should plot the distribution of angles (e.g. in a rose diagram).

Unfortunately, our ability to image divisions is restricted by the slow growth of the system. We collected nine complete divisions (starting from interphase) in over 40 hours of imaging, with approximately 100 dissections. During our revision period, we collected an additional division. We agree that this low number is problematic and have therefore extended our analyses to divisions in the embryonic neuroectoderm, a tissue that develops

more rapidly. We have added a figure showing the difference between the interphase long axis and final division angles for both the FE and ENE (Fig 4G).

We find that in our hands rose plots can amplify negligible differences (depending on the binning) and we have therefore stopped using them.

-Hertwig rule does not mean that all cells divide exactly perpendicular to their interphase long axis, but that there is a bias towards this division orientation. The question is therefore how strong (or weak) this bias is in the follicle epithelium as compared to other epithelia that have been previously analyzed (e.g. Bosveld et al, 2016).

Hertwig's Rule (1884) has been translated into English as follows: "The two poles of the division figure come to lie in the direction of the greatest protoplasmic mass." (Minc, Burgess, and Chang, *Cell* 2011 and Gillies and Cabernard, *Current Biology* 2011). The "Hertwig rule" article on Wikipedia (July 26, 2018) begins as follows: "Hertwig's rule or 'long axis rule' states that a cell divides along its long axis." We believe that our use of the term in our manuscript is consistent with these definitions.

Fig. S5F It is unclear how the conclusion that the distribution of cell vertices does not correspond to division orientation is reached.

We apologize for the lack of clarity. Our original figure S5F showed that the final angle of division orientation is random with respect to the distribution of cell vertices calculated at metaphase. We have developed an automated Matlab script during the revision period and expanded our analysis of the vertex distribution versus the final cell division angle at both interphase (Fig 5A) and metaphase (EV5A) cells in both the follicular epithelium and the embryonic neuroectoderm. At both interphase and metaphase, and in both tissues analyzed, the final division angle is random with respect to the final division angle. The mean difference in angles is approx. 45^{o} in all cases with a wide standard deviation.

The distribution of cell vertices was calculated as follows:

Images taken from live movies of egg chambers or embryos expressing the membrane marker Bsg-YFP together with Tub-RFP and Cnn-RFP to mark the position of spindles. Images were segmented and the positions of cell barycenters, vertices and the vectors from the barycenters to the vertices were identified using a custom MatLab script. The unit vectors from the barycenter to the vertices of the cells were used to calculate the expected division axis ('Vertex Bipolarity') by use of the TCJ bipolarity matrix as defined in Bosveld et al (2016):

$$B = \sum_{n=1}^{N_{TCJ}} (\hat{u}_n \otimes \hat{u}_n)$$

where B is the TCJ bipolarity Matrix, N_{TCJ} is the TCJs, and $\hat{u}_n$ refers to the unit vector to the n^{th} TCJ. The principal eigenvector of the TCJ bipolarity matrix defines the predicted axis along which the cell will divide, while the relative difference of the eigenvalues helps to determine how favored this axis is compared to the orthogonal direction (not shown). We also considered the possibility that the distance of vertices from the barycenter could influence the magnitude of the pulling force. To do this, a similar calculation of the TCJ bipolarity matrix was employed, Modified Vertex Bipolarity, with the only caveat being that the full vector was used as opposed to the unit vectors.

We have expanded our Materials and Methods section explaining how the vertex bipolarity values are calculated.

-Fig. 6A,B. The authors claim that Mud (the authors in fact look at Mud-GFP) localize around the entire mitotic cell cortex in the follicle epithelium, in contrast to the pupal notum where Mud is enriched at tricellular junctions (yet detectable around the entire cell cortex (Bosveld, 2016)). The pixel intensity appears saturated in Fig. 6A,B and I am

wondering whether the authors are therefore missing an enrichment at tricellular junctions. Better images are required.

1) Out of concern for the quality of our live imaging (formerly Figure 6A,B, now 5B,C), we included a higher-resolution fixed image (formerly Figure EV6D, now EV5D) in our original submission. We trust that this image is of sufficient quality to allay the reviewer's concern.

2) The Mud-GFP line used in our work was developed for and used in the study cited by the reviewer (Bosveld et al, Nature 2016).

3) The last author of our manuscript (Dan Bergstralh) observed and reported the same localization for Mud at TCJs in the imaginal wing disc that Bosveld *et al* observed in the pupal notum (Bergstralh et al, Development 2016). We believe that Dr. Bergstralh, who performed the imaging, is unlikely to have overlooked a similar enrichment in other tissues.

4) As discussed in our original submission, specific enrichment of Mud-GFP at tricellular junctions would be unexpected in the follicle epithelium and early embryonic ectoderm, since tricellular junctions are dedicated protein structures not observed in either tissue.

-Fig. 6D. I suspect that the authors have plotted the angle of spindle rotation. The authors state that they have sometimes observed oscillatory behavior. How is the angle of spindle orientation defined under such behavior? Moreover, I cannot follow the authors' argument that differences in the magnitude of spindle rotation suggests differences in the way the orientation of division is determined.

We have removed the data describing differences in the magnitude of spindle rotation.

-Fig. 7B. The authors need to test whether canoe shRNA specifically targets canoe, e.g. by using at least two additional independent shRNA lines. Or even better, but perhaps more difficult, to analyze planar division orientation in cno-R2 mutant clones that the authors have generated. Moreover, is the angle distribution between wt and random number generator significantly different?

1) The shRNA line that we used was generated by the Transgenic RNAi Project. Only one shRNA that targets Canoe is available from the TRiP, though there are two distinct lines that use different promoters. We used the promoter that is optimized for somatic tissue in our study. During the revision, we repeated this experiment using the other line, which uses a promoter optimized for germline expression, and saw a less pronounced effect on spindle orientation (average angle of 41 degrees, $n = 39$ divisions) as well as a weaker knockdown as measured by protein expression. We consider this result to be a weak control that is not very meaningful, and we have therefore not included it in our manuscript. We regret that additional alternative lines could not be developed within our revision period.

As the reviewer suggests, the difficulty of using mutant clones in this experiment is prohibitive.

2) As stated in the text of the original submission (page 14), the wild type "division angle data [is] significantly different from a hypothetical random data set."

-The authors would need to show some more direct evidence that tissue tension directs cell division orientation. Wang et al 2017, for example, used laser ablation to mechanically isolate cells in the Drosophila embryo. These cells normally divide in an oriented fashion, but when mechanically isolated divide with random orientation. Similar experiments would need to be done in the follicle epithelium.

Development of the follicle epithelium is much slower than the embryonic ectoderm. We also find that the bias is slight – less than 15 degrees away from random – and division orientations are widely distributed. These observations inform our model. Given the weakness of the bias and the relative infrequency of cell divisions in the follicular

epithelium, it would require more than three months of work to perform a meaningful comparison between ablated and wt conditions. Furthermore, our analyses performed on the behavior of mitotic cells in the embryonic ectoderm are consistent with findings from the follicular epithelium. As ablations have already been performed in the embryo by Wang *et al*, the results of another experiment in the ovary may not justify the effort.

Minor concerns

- Fig. 1C: The authors claim that cells are consistently smaller by stages 6-7. However, smaller appears to relate to apical/basal area, not volume (cells could change their height). The conclusion that proliferation outpaces elongation (of the egg chamber?) seems therefore not adequate.

The reviewer raises an important point. We have measured a decrease in cell surface area at the apical and basal surfaces as the egg chamber increases in size. Since the egg chamber is edgeless, the change we measured demonstrates an increase in cell number regardless of individual cell volume. We have edited the paragraph to clarify as follows:

"We used automated segmentation and image analysis to measure features of cell and tissue geometry (Fig EV1A) and found that follicle cells decrease in cross-sectional area as egg chambers elongate (Fig 1C). Because the follicular epithelium is edgeless and expands as it develops, this result shows that the number of cells per unit tissue area is reduced, and therefore that cell proliferation outpaces organ elongation."

-Page 6 top: What does 'loosely organized state" mean? The authors need to be more precise.

We used unbiased algorithms to quantify cell shape, cell size, and cell sidedness at large scale in our study. We hope that the reviewer finds these measurements sufficiently precise. We have chosen to use this language to summarize the data in an attempt to help the reader.

- Page 6 top: "Together, these results show that the FE is proliferative and dynamic.." These conclusions are quite general and not novel. I do not see evidence that "the material properties of the tissue change..." The authors should formulate the conclusion more precisely. Moreover, two paragraphs later, the authors write "The relatively static arrangement of cells..." Wording needs to be improved.

We agree with the reviewer and have abbreviated these paragraphs to improve clarity.

Fig. 2A,B. The authors claim that knockdown of string does not affect egg chamber elongation. The authors should measure the aspect ratio for string knockdown and control egg chambers for different stages.

The data presented in our original Figure 2B shows that the relationship between aspect ratio and egg chamber volume (a more precise measurement than developmental stage) is unchanged by the String knockdown. We have added the developmental stage comparison to Fig EV2.

-Fig. 2C: Does the cell shape regularity differ between controls and string knockdown? Statistical test is required.

The data are statistically different at AR 1.2-1.4 ($p = 0.0029$) and AR 1.4-1.6 ($p < 0.0001$) using an unpaired t test with Welch's correction. We have added the p values to the figure and amended the legend accordingly.

-Fig. S2C: I do not see a gap where the arrow is pointing to.

We apologize for not making this gap clearer. The arrow in our original Figure 2C (now 2D) pointed to a germline cell that expanded to take the place of the follicular epithelium. We have moved this arrow so that it points to the start of the gap and amended the figure legend accordingly.

-Fig. 3C: The distribution of Sqh at the basal site of cells, as shown in the lower panels, is not mentioned in the results section.

We regret for the omission. We have amended the text to explain these data, now shown in Fig EV6F.

-Page 10. The authors claim that the rate of proliferation is constant over the tissue, but do not show data. Either the authors show the data or do not make this claim.

These data are now shown in Figure EV4A.

-Fig. 5B. The authors claim that dividing cells detach and are excluded from the basal surface. I do not see this. In fact, in the basal view there is a tiny cell in the center of the image. Is this the basal foot of the dividing cell? The authors need to label single dividing cells (e.g. by expressing GFP in single-celled clones) to clarify this issue.

Detachment from the basement membrane has been previously reported using different markers (Bergstralh*, Lovegrove*, and St Johnston, NCB 2015). Our observation in the current paper is not that cells can become detached, since that is established, but rather that other cells can fill in the space. A ghost outline representing the “shadow” of the dividing cell may be apparent in our image because of the limit of confocal resolution. However, the boundaries of the neighbors are very clear. Additionally, we included an orthogonal view in the original submission, and can tell from that image that the cell does not have any substantial connection to the basement membrane, though the possibility of a thin process (rather than a foot) cannot be excluded.

-Fig. 5C. The authors claim that the cell shape bias is the same at apical and basal cell surfaces. This panel shows a segmentation, but I do not see that apical and basal surfaces of a cell are compared.

As the reviewer notes, we did not compare the apical and basal surfaces of individual cells. We compared the bias in cell shape at the apical and basal surfaces over a large scale, and found that these biases are the same. We have clarified this point in the text.

Suggestions

-Abstract: The abstract should include a few sentences of background information.

We appreciate the reviewer’s suggestion, but we are restricted by the word limit.

- Title: The authors investigated cell division in the Drosophila follicular epithelium. This should be indicated in the title.

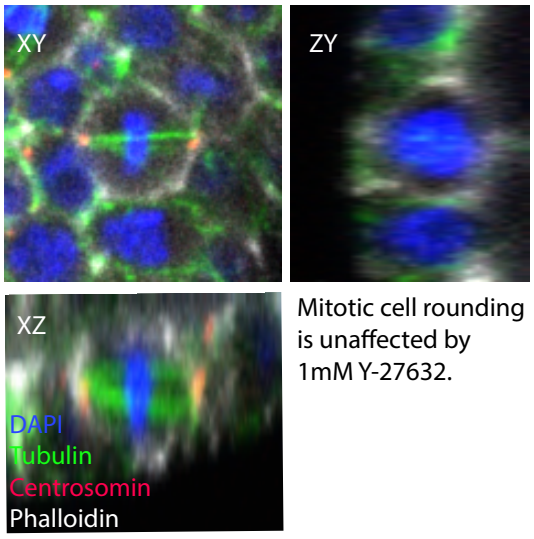
We prefer the title: ‘Tissue tension and not interphase cell shape determines cell division orientation in the *Drosophila* follicular epithelium’ but this exceeds the character limit for the journal.

- Possible typos:
- page 8 bottom: "This raises suggests.."

Thank you. We’ve fixed the typo in our resubmission.

Referee #2:

MAJOR
1. The authors show that inhibiting Rok with Y-27632 does not affect cell division orientation. They should try to further increase the concentration of Y-27632 until they observe myosin displacement from the cortex, and thus can confirm that a) myosin is not necessary for cytokinesis, and b) myosin does not affect division orientation. Otherwise, how do they reconcile the fact that cell division orientation is reduced in cno loss-of-function, but not in Y-27632? If not through myosin, how does then Canoe control cell division orientation? This is a major outstanding issue.



Mitotic cell rounding is unaffected by 1mM Y-27632.

We treated egg chambers with Y-27632 and examined its effect on mitotic cell rounding and on cytokinesis, which are Rok-dependent processes. Our experiments were performed using manually-dissociated ovarioles treated for 45 minutes in Schneider Cell Medium supplemented with insulin. Even at our maximum concentration of 1mM Y-27632, several orders of magnitude above the K_i, we are unable to see any effect on mitotic cell shape or actin accumulation at the mitotic cortex (left) and did not observe any evidence of defective cytokinesis. We also did not observe defects in spindle organization or orientation, as would be expected from failed rounding. We therefore do not believe that the inhibitor works in this tissue, and have removed the data from our manuscript. One possible explanation is that the follicle cells are secretory, and likely to pass molecules taken up from the media quickly into the germline.

We are unable to reconcile our results with work from Alégot et al that showed a modest effect of Y-27632 on apical pulsing in the follicular epithelium. Egg chambers have also been treated with Y-27632 in another study (Schotman et al), though in that work the same effect was observed for Blebbistatin, which is ineffective against *Drosophila* myosin.

2. Figure S5D-E: I have several issues with these figures:

a. The rounding of Fat2 shRNA is significant, but the chamber is still significantly elongated. Are there other treatments that result in rounder chambers and could be used to confirm these results? Fat is a key component of the planar cell polarity (PCP) pathway, and I worry that the phenotype in cell division orientation could be due to a PCP defect.

The reviewer raises an important point. To address this concern, we have added quantification of the cell division angles from egg chambers in which the protein Pak was disrupted by RNAi. As previously reported, these egg chambers are substantially rounder than *w*- and Fat2-shRNA egg chambers (quantified in Fig EV4C). Spindle orientation at Stage 6 is random (Fig 4). However, this experiment is somewhat difficult to interpret, given that Pak is reported to regulate actomyosin contractility.

b. What is the evidence that cell division orientation in Fat2 shRNA is independent of cell shape cues? In Fat2 shRNA chambers, the cell shapes also lose their preferential orientation perpendicular to the long axis of the chamber, right?

This is a good point. The evidence that cell division orientation is independent of cell shape cues comes from the control experiments. We found that cell long axes align towards the spinning direction before divisions are reliably oriented, as shown in Figure 4B.

c. The authors claim that anisotropic tissue tension is lost in Fat2 shRNA, but they should use their laser ablation system to demonstrate this. Based on the significant elongation of Fat2 shRNA chambers (Fig S5D), it is likely that tension is still anisotropic.

We would ideally like to measure tissue tension in rounder egg Stage 6 chambers by performing laser ablation on chambers that have been genetically modified for *fat2* or *pak* at Stage 6. We attempted to generate these flies but were unable to recover ovaries. We attribute this problem to the complexity of the background, as described in our response to the first reviewer (above).

We have performed ablation experiments on rounder egg chambers at stages 4 and 5 with aspect ratios of less than 1.3. In these egg chambers, we found that recoil in the elongating (AP) versus the rotation direction (UD) was equal on average (Mean ratio of recoil AP vs. UD = 0.9871; Median = 0.9884; Wilcoxon Signed Rank Test compared to theoretical median of 1 p = 0.8438). These data, presented in Fig EV4E, indicate that tension anisotropy is reduced in rounder egg chambers, consistent with our model and spindle orientation results.

3. The authors use laser ablation to probe the mechanical properties of the apical surface of FE cells. It was not clear to me, based on the description of the methods, whether they are using a two-photon system (it could be, based on the wavelength used for ablation). Regardless, because apical is "deep" in this tissue, and equator cells are flatter than pole cells, it is possible that the differences in recoil velocity could be explained by a difference in the ability of the laser to reach the apical surface of polar vs. equatorial cells. Given that the authors find similar cell shape changes apical and basal (Fig 1D), they should try to confirm their results conducting laser cuts on the basal surface, which should remove any concerns about laser penetration.

We apologize that the systems used for the ablation work were unclear and we have clarified this in the Methods section of the manuscript. To test the difference in tension at the equator versus the poles of egg chambers we used a two-photon system. We performed the experiment with the egg chambers in sagittal view, measuring recoil along the plane of the apical surface of the egg chamber (Fig 4F) and therefore did not move the plane (depth) of imaging between our cuts at the equator and the poles for this experiment. Because our imaging is done halfway through the egg chamber, differences in the amount of tissue through which the laser travelled at these two sites should be negligible.

We did attempt to perform laser cuts at the basal surface as a control but could not see evidence of measurable recoil by imaging basal Sqh-GFP fibers.

We performed all of the round ablation experiments used to measure the difference between tension in the elongating (AP) vs. rotating (UD) axes at the equatorial position roughly mid-way along the length of the egg chamber at the flattened apical surface. Hence, depth should be consistent between these experiments.

We carefully assessed each cut for evidence of improper laser density or pulse length to assess if correct photoablation-induced cutting of the cortex occurred (at low power values the sample simply is bleached, whereas at high values, plasma induction and photodisruption is observed) and we only included those experiments which exhibited clear evidence of recoil and recovery following ablation (observed only approx. 1 min time scale).

4. Figure 3A-B: using what seem like low magnification images of egg chambers, the authors argue that the levels of beta-catenin and non-muscle myosin do not change in the FE as the chamber elongates. However, there is no quantitative analysis to support that claim. In fact, based on Figure 3B, I would argue that I first see an increase and then a decrease in apical myosin fluorescence. The authors should provide quantitation of beta-catenin and myosin fluorescence to back up any statements about levels.

We agree with the reviewer and have therefore removed these claims from the manuscript.

5. What is the importance of hexagonal packing? How beneficial (and why) is it for the embryo to obtain the relative increase in hexagons observed in Figure 8B (left) with respect to what you would get if divisions followed Hertwig's rule (Figure 8B, center), or were randomly oriented (Figure 8B, right)? In other words, what is the advantage of the final cell packing in wild-type chambers vs. cno shRNA? This should at least be discussed.

We don’t know if hexagonal packing is important to this tissue. However, hexagonal packing maximizes regularity of the tissue and the density of packing. This may help to

minimize differences in tension across the tissue. Cno shRNA egg chambers exhibit a reduction in hexagonal packing, but preliminary data suggests that they recover seemingly normal tissue geometry following Stage 6 (this is presumably due to cell intercalations which have recently been reported to occur from stage 7 onwards). We have rewritten the manuscript to de-emphasize the focus on hexagonal packing.

MINOR

1. Figure 3C: are these cells in the poles or the equator of the egg chamber? Are there any differences in the distribution of apical myosin between polar and equatorial cells? This is important for the interpretation of the laser ablation experiments.

We apologize for not making this point clearer in the original manuscript.

Since we did not position any egg chambers such that the poles were flattened up against the cover slip, we cannot rule out that the cells at the extreme poles exhibit differences in the localization of Myosin. Alégot et al performed imaging of Myosin specifically at the poles but did not report any differences in the localization pattern.

2. Figure 4D, E: the authors report the ratio of polar/equatorial cell heights, but it would be useful to see the absolute values of these heights to really understand how much the tissue is flattening.

We've added these measurements, which are now shown in Figure EV3A.

3. Figure 5A: the authors plot the orientation of cell division with respect to the long axis of the embryo (i). But wouldn't it make more sense to plot the orientation of cell division with respect to the axis of tissue expansion (shown in ii). Wouldn't those correlations show the bias of cell division orientation in the direction of tissue expansion a lot better?

Yes, they would. We love that idea and regret that we haven't shown the data that way. Unfortunately, the tool that we use to measure spindle angles was developed before we started to think about tissue expansion angle as an important parameter. Because of the mathematics, we can't simply replot our existing data. Instead we would have to rework the tool reanalyze the data. These analyses require substantial computing power (we had to purchase a dedicated machine for this work), person power, and time. We focused our efforts on performing suggested experiments instead.

TYPOS

1. Page 4, paragraph 3: the reference to (Fig 4F, G) should be a reference to (Fig 4D, E).

We've corrected the reference.

2. Page 4, paragraph 3: "This raises suggests two possibilities" has too many verbs!

We've corrected the sentence.

3. Page 14, last sentence: "Adherens junctions are not proximal to spindle poles in this tissue (Fig S4C)". Fig S4C shows a recoil speed after laser ablation. Is that the correct figure reference?

It was not. Thanks. We've corrected the reference.

4. Page 15, paragraph 2, line 6: should the reference to Fig 5A be a reference to Fig 7B?

Yes. Thank you. We've corrected the reference.

5. Page 22: please review this sentence "Cuts in the saggital view were performed on an Olympus FV1000MPE and an Olympus Fluoview FVMPE-RS Twin Lasers Gantry System using an Olympus 25x 1.05NA objective lens which using an Insight X3 laser."

Thanks. We've corrected the sentence.

Referee #3:

Major issues

p. 5 and onward. Throughout the authors assess parameters of cell shape, but in the text do not tell us which molecule they were visualizing,

Apologies. We have now included this information in in body of the manuscript. We used Basigin::YFP to assess cell shape.

Fig. 2. The authors state that string knockdown does not change aspect ratio but do NOT actually show the data. A graph of aspect ratio should be added. They also state that "proliferation outpaces growth" and bolster this by assessing cell area-however, without an independent assessment of cell height (which I imagine they made), I am not sure how they can conclude this.

1) The data presented in our original Figure 2B shows that the relationship between aspect ratio and egg chamber volume (a more precise measurement than developmental stage) is unchanged by the String knockdown. We have added the developmental stage comparison to Supplemental Figure 2.

2) The follicular epithelium is edgeless and increases in total area throughout development. The decrease in individual cell areas therefore means that there are more cells per unit area, regardless of cell height. (We have also added cell height measurements, which are shown in Figure EV3A.)

p. 7, last paragraph. The authors measurements of Arm and myosin protein levels do NOT, by themselves "refute the possibility" that there is an increase in junctional tension. This needs to be tempered.

We have removed these claims.

The one place where the data did not match the conclusions was in their assessment of planar polarity of important proteins. They present images of Ecad, Cno and Jub and state there is "no obvious planar polarity". These data have two issues. First, in the image they show Jub looks clearly planar polarized. Second, planar polarity is seldom "obvious". They should quantify levels of each on A/P vs. D/V borders. In the extending germband, for example, planar polarity of Ecad is less than 2 fold, and not immediately obvious by eye. I think these potential differences are very interesting and fit with their later analysis of rhe role of Cno.

We take the reviewer’s point seriously and are very interested in this aspect of the biology. We have added intensity quantifications for Shotgun, Canoe, Armadillo and Ajuba across junctions (Fig EV6A-D) at Stage 6. We did not observe planar polarization at a tissue level, at least at our ability to detect. (We do not expect to see polarization comparable to that observed during germband extension, which is orders of magnitude faster than egg chamber elongation). However, our preliminary results suggest that mechanosensors may exhibit planar polarization in mitotic cells. We are currently following up on these observations.

Fig. 4D,E should also include a graph of actual cell height as well as the ratio shown.

Cell height measurements are now shown in Figure S4A.

Fig. 4F-I and p. 9 middle. I was confused by the explanation of the laser cutting experiments. In the text the authors suggest they could not measure "recoil speed" yet that is exactly what is reported in Fig. 4G. This needs to be better explained.

We apologize for the poor wording of this paragraph in the original submission. We have simplified the sentence to remove ambiguity. Our original statement was meant to convey that the spatial and temporal resolution of our instrument did not permit a detailed analyses of recoil dynamics.

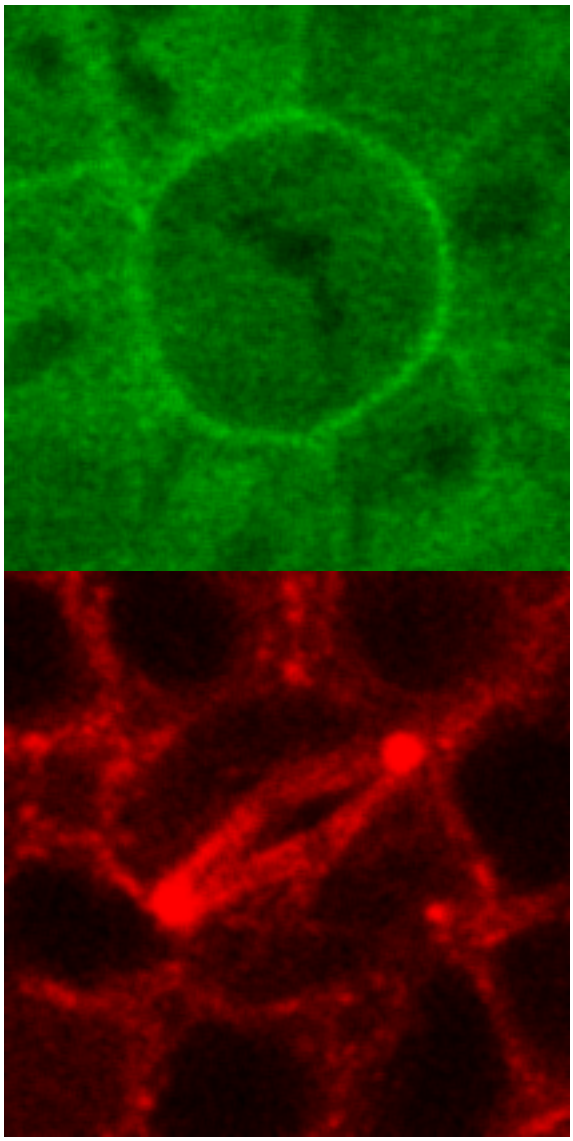
p. 12, top. The quantification of divisions live reported here was important but the authors

need to describe what the data mean-what would be the predictions if there was no bias and how do these data refute that.?

We have now a figure showing the deviation of cell division angles from the angle predicted by the cell long axis (Fig 4G) and expanded our discussion of these results.

p. 13 and Figure 6. I found the description of the vertex model confusing. I also though some of their assertions were not accurate. In Fig 6A Pins appears to be enriched at vertices, while they suggest otherwise.

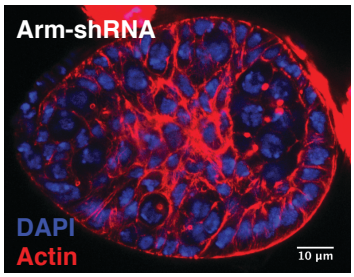
We do not see much vertex enrichment for Pins. We can see three brighter yellow spots in Figure 6A where Pins and Tubulin colocalize, and at least one of these is a vertex. (The two original channels are copied below). We can also see vertices at which Pins is clearly not enriched. To clarify the localization of Pins even further in our manuscript we have added another image, Figure EV5D', which shows Pins-YFP alone.



I also had trouble with their descriptions of the events around spindle rotation. Do their data rule out a model where a cortical cue captures spindle poles as the cell transitions from metaphase to anaphase? I think the conclusions in this section are too strong.

We agree and have rewritten the paragraph to soften our conclusions.

p. 15, top. The authors saw no effect for Ecad KD, presumably due to Ncad's presence. Did they try Arm KD?



We agree that there is probably no effect for E-Cad KD due to redundancy with N-Cad. We attempted to analyze the effect of Arm KD using shRNA but in the rare cases where we could find egg chambers, we were unable to obtain intact epithelia (example shown left) in order to perform analysis.

p. 16, Fig 8. The use of mathematical modeling is a significant strength. However, the brevity with which this data is described makes it difficult to assess the match of model and data.

To address this point, we include more detail in our description of the mathematical model in the Material and Methods, in particular with regard to the development and parameterization of the model and its quantitative comparison with our *in vivo* data. We note that the model was developed independently of our data collection, using idealized parameters, and not "tuned" afterward to fit the data.

Text and data clarity issues

p. 4. The authors first introduce Hertwig's Rule and the Tricellular junction rule here, but do not define either for the reader who is not in the field.

Thank you for highlighting this. We have now expanded the preceding paragraph on page 4 to introduce these rules.

p. 5, top. In the 1st sentence of the results the authors should be clearer, stating that they examined geometry "during the proliferative stages, as the egg chamber aspect ratio increases from ~1.0 to 1.6" and then annotate this on Fig. 1A.

We have amended the text as the reviewer suggests.

Fig. 1C-E, p. 5 middle. Here is where the authors begin their quantitative analysis. They need to go much more slowly, explaining to the reader what they measured and what it means. These data underlie one of their important conclusions-that proliferation increases cell shape regularity.

We have expanded and rewritten the text in this section to improve clarity.

Fig. 3C is very interesting and important yet complex. It needs more interpretation for the reader, annotating cell border and apical staining. I'd also like to see a still from stage 6 in which both myosin and a junctional marker were included to see how the supercellular structures align with cells. Finally, it would be better to conclude that the changes in Sqh localization "coincides with" rather than "corresponds to" the change in tissue regularity

We appreciate these suggestions and have now the localization of Sqh at the apical cortex relative to cell-cell junctions by including images of Sqh-mCherry and Shg-GFP at the apical cortex at different stages (Fig 6B). We have also included a higher-resolution confocal image of Zip-YFP at the apical cortex which exhibits the same localization pattern through development as observed for Sqh-GFP (Fig 6A). We have followed the reviewer's suggestion and changed "coincides with" to "corresponds to" in this sentence.

Fig. 4A-C is described in a single sentence!

We've rewritten this section to clarify.

p. 9, top paragraph. I had a difficult time following the logic in which observations of uneven cell height led to the conclusions about mechanical deformation-explain better or remove.

We consider this observation to be an important one. We have rewritten the paragraph to

clarify the logic.

p. 10, middle. Where is the data reporting PH3 immunoreactivity mentioned here?

These data are now shown in Fig EV4A.

p. 10, bottom and Fig. 5A. These data are key to one of the conclusions and once again need to be more thoroughly explained. Define what 0 degrees means-perhaps with a vector diagram of zero versus 90 degrees added to the top of this panel/. . Place a line at 45 degrees as that would be the predicted mean of random divisions.

We have addressed this comment by including a cartoon (now Fig 4A) to illustrate how the expansion angle was calculated.

P 12, middle. The section starts with a bold statement about the uncoupling of cell shape and tension that I was not able to simply connect to the preceding data.

We agree with the reviewer's comment and have rewritten the sentence.

p. 14, bottom. Canoe ≠ adherens junctions, and the sentence should read "Canoe is not enriched proximal to..." (and the ref. should be to Fig. S6C).

Thank you for this clarification. We have amended the sentence as suggested and changed the figure reference.

p. 15, bottom. Please do not describe the effect on myosin with the term "drops away". Earlier authors have described this as an effect of Cno loss on the linkage of actomyosin to cell junctions.

We apologize for the poor wording of this sentence. We have removed 'drop away' and rewritten the paragraph.

p. 16, top. The logic of this paragraph was totally lost on me.

We've removed the entire paragraph.

Minor issues

p. 4, top. The FE rotates, while the underlying germline does not, so I would be careful to not simply say the "egg chamber rotates". Likewise, state the divisions are oriented "perpendicular to the apical-basal axis".

The underlying germline rotates along with the epithelium (Haigo and Bilder, 2011), so we are comfortable with our wording.

p. 5, bottom. Cells do not "get smaller"-apical area does.

This is an important distinction and an error on our part. We have amended it in the current draft.

Fig. 2A. Tell us what stage is displayed.

We have added this information to the figure legend.

p. 8, bottom. In the last paragraph a Figure reference is incorrect-it should be "anterior and posterior poles (Fig. 4D,E)."

We have corrected the sentence.

p. 13, top. Temper this conclusion. "This observation may be explained by..."

We have amended the sentence as suggested.

p. 15. Several Suppl. Fig refs. here are wrong.

We apologize and have fixed our referencing.

2nd Editorial Decision 23rd Oct 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by all of the original referees whose comments are shown below. I apologize for the delay in getting back to you, it took longer than expected to receive the full set of referee reports.

As you will see, referees #2 and #3 find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. Referee #1, on the other hand, has remaining concerns mainly regarding the low number of cells analyzed to challenge Hertwig's rule and need of a more direct test to indicate the link between anisotropy and orientation of cell division. I have taken another detailed looked at everything and noted that these points were already responded/discussed in a way that I - as well as the referees #2 and #3 - find satisfactory.

However, before I can officially accept the manuscript, there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS:

Referee #1:

The manuscript by Finegan et al. has addressed some of my concerns. However, I still believe that the major claim that tissue tension, and not interface cell shape, determines cell division orientation is not well supported. One difficulty with this study is that cells in the follicle epithelium divide rarely. The authors are able to observe only 10 cell divisions by live imaging and are able to analyze the relationship between interphase long axis and division angle (now Fig. 4). I do not think that with this small number of analyzed cells the Hertwig's rule can be challenged, as claimed by the authors (Abstract: Hertwig's Rule, [...] is therefore broken in this tissue".) A second challenge is that the anisotropy of tissue tension is weak (now Fig. 3). The authors show some correlative evidence between tissue anisotropy and cell division orientation (now Fig. 4), but a more direct test of whether this anisotropy directs cell division orientation is missing. Finally, the authors have done little too improve the writing of the manuscript. The manuscript still lacks focus reporting too much data that is not directly relevant to the main question of the manuscript (e.g. Figs. 1-2).
In conclusion, while the manuscript does report on novel findings, given the weaknesses I do not think that it is of sufficient general interest to merit publication in EMBO J.

Referee #2:

The authors have addressed most of my concerns (perhaps with the exception of replotting the orientation of cell division data with respect to the axis of tissue expansion rather than the long axis of the tissue). I support publication of the manuscript.

Referee #3:

As I noted in my initial review, the proper regulation of organ shape is a key question in cell and developmental biology. Cell proliferation, migration, and shape change all have the potential to play important roles. The Drosophila ovary has emerged as a superb model, and analysis of its morphogenesis has already revealed a number of very striking features, including a fascinating collective migration event. Building on this foundation, the authors explore earlier events in ovary morphogenesis. They use a very impressive set of quantitative approaches to comprehensively assess the contributions of proliferation, migration and oriented cell division to the final ovariole shape. They find that cell proliferation and increased packing increases cell shape regulatory, and identify a surprising and exciting role for tissue wide biases in myosin-based tissue tension in directing oriented cell division in a way that defies Hertwig's rule, which governs cell division in most contexts. They couple direct observation with mathematical modeling to assess this hypothesis. The results are well documented, carefully quantified, and support their conclusions. In my initial

review I had some issues with clarity of the approaches and with their analysis of a subset of the data. The authors have done a substantial amount of new work, analyses and text clarification in response to the comments of myself and all of the other reviewers. These changes fully address all of my issues and, in my opinion, also address the thoughtful comments of the other reviewers. I think this story will be of broad interest to cell and developmental biologists, changing our view of how oriented cell divisions shape tissue architecture, and is well suited for publication in the EMBO Journal.

3rd Editorial Decision 2nd Nov 2018

Thank you for submitting the revised version of your manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication at The EMBO Journal.

Congratulations on the very nice work!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dan Bergstralh
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-100072

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We used Excel to generate a random distribution of hypothetical division angles (1-90) and tested for deviation from random.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used small invertebrates (flies) and were not restricted by availability.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, data analysis was performed by undergraduate students blind to the genotypes and to the hypotheses tested. When blinding was not possible, analyses were performed by at least two researchers working side-by-side. We also developed automated analysis scripts to further minimize the possibilty of human error.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We chose statistical tests that fit the properties of the data. We used Prism software to help us with the analysis.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	We represented each individual data point in our graphs and included error bars to indicate the standard deviation from the mean.
Is the variance similar between the groups that are being statistically compared?	Variance is similar, and we used Welch's correction as appropriate.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information is included in our Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We used small invertebrates (flies) and have described them as appropriate. We dissected only adult female flies for this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	The computational modeling software (Chaste) used in our study has been previously described and is available.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA